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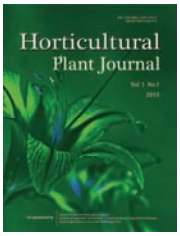

The Effects of Polyphenol Oxidase and Cycloheximide on the Early Stage of Browning in *Phalaenopsis* Explants

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Abstract

Explant browning is one of the major problems in the tissue culture process, and polyphenol oxidase (PPO), is the major proteases involved in plant tissue browning. We investigated the effects of polyphenol oxidase on the early stage of browning in explants of the orchid *Phalaenopsis*. Our results show that PPO activity was significantly higher in explants cultured for 3 d than in the 0 h control. The levels of *PPO* transcripts and PPO protein were significantly higher in explants cultured for 6 h compared to the 0 h control; these high expression levels were maintained over increasing cultivation time. Cycloheximide (CHX) treatment reduced *PPO* transcript levels, PPO protein levels, and PPO enzyme activity. High levels of *PPO* mRNA and PPO protein were detected in the cytoplasm and vascular bundles of *Phalaenopsis* explants cultured for 6 h compared to explants cultured for 0 h, 24 h, and 3 d. CHX treatment did not significantly affect the distribution of *PPO* mRNA and PPO protein in explant tissues, but their levels were significantly lower than those of the untreated control.

Keywords: *Phalaenopsis*; PPO; explant browning; CHX; gene expression

1. Introduction

Enzymatic browning of plant tissues causes unappealing changes in fruits and vegetables such as cut apples and potatoes, and can cause explant death and failure of regeneration in explant culture for propagation of ornamental species such as orchids. Enzymatic browning mainly results from the oxidation of polyphenols to quinones, catalysed by browning enzymes such as polyphenol oxidase (PPO, EC 1.14.18.1) (Luo et al., 1999). PPO activity *in vivo* typically occurs in damaged plant tissues that have lost cellular compartmentalisation. The total phenolic content, as well as the expression and activity of PPO increase during tissue browning in apple and litchi (Leng et al., 2009; Di Guardo et al., 2013, Wang et al., 2014). Overexpressing *PPO* in transgenic sugarcane results in seriously browning and an increase in PPO content in the sugarcane juice (Vickers et al., 2005). Potatoes and apples with low levels of *PPO* expression exhibit low rates of browning and a delayed onset of browning (Bachenm et al., 1994; Murata et al., 2000; Coetzer et al., 2001; Arican et al., 2003; Wang et al., 2007). Using artificial microRNA technology, Chi et al. (2014) found that reducing the expression of *StuPPO1* and *StuPPO4* reduced the browning rate in transgenic potatoes. These findings help confirm the notion that the expression of *PPO* affects tissue browning.

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During tissue culture of apple (Murata et al., 2001) and Cangxi pear (Li and Yan, 2001) explants, browning rate is associated with PPO activity and total phenolic content. Using antisense RNA technology, an antisense *PPO* DNA sequence was transferred into apple by *Agrobacterium rhizogenes*-mediated transformation. The resulting transgenic callus exhibited a low browning rate, which is consistent with low PPO activity (Laukkanen et al., 1999), suggesting that PPO is involved in the browning of explants. PPO activity also increases during the browning of *Phalaenopsis* explants (Xu and Li, 2006). Analysis of PPO isozymes in *Phalaenopsis* revealed new PPO isozyme bands that occurred before explant browning; this isoform also had higher PPO activity (Xu and Li, 2006). The PPO activity in *Phalaenopsis* explants with severe browning exceeds that of explants with slight browning (Zhao et al., 2006; Huang et al., 2007). The browning of *Phalaenopsis* explants can be controlled, to some extent, by inhibiting PPO activity (Chen et al., 2009; Lai et al., 2010). These findings suggest that PPO activity is correlated with the browning of *Phalaenopsis* explants. Unfortunately, little attention has been paid to the factors initiating explant browning and to the underlying molecular mechanisms. The time period during which PPO influences the occurrence of browning is difficult to determine at the physiological level. Moreover, PPO in plants is in pro-enzymatic form (Gooding et al., 2001; Michael et al., 2009), making it important to determine whether explant browning is induced by PPO pro-enzymes and/or if PPO enzyme is biosynthesised later in the browning process.

The orchid *Doritaenopsis* Queen Bee 'Red Sky' is a *Phalaenopsis* hybrid that readily undergoes browning in tissue culture. We previously cloned the full-length *Phalaenopsis PPO* gene (GenBank accession number: EF363553.1) and obtained PPO antibody (Xu et al., 2009), providing key reagents for investigating the effects of PPO on explant browning at the molecular level. In the current study, we treated *Phalaenopsis* explants with the protein synthesis inhibitor cycloheximide (CHX) and analysed *PPO* transcript levels and PPO protein levels during the early stage of browning, in the first three days of culture. We also examined the effects of the timing and position of *PPO* expression on the occurrence of explant browning. These experiments helped us determine whether newly synthesized PPO or existing PPO causes explant browning. The results of this study increased our understanding of the mechanisms and occurrence of explant browning and may be helpful for designing methods to effectively control browning in orchid explants.

2. Materials and methods

2.1. Preparation of plant materials

Phalaenopsis (*Doritaenopsis* Queen Bee 'Red Sky') seedlings were grown on Murashige and Skoog (MS) medium containing active carbon (1 g · L^{-1}). Leaves of *Phalaenopsis* seedlings were cut into 0.5 cm × 0.5 cm segments and immediately transferred to fresh MS medium containing 6-benzylaminopurine (3 mg · L^{-1}) or supplied with CHX (0.1 mg · mL^{-1}) (Zhou et al., 2000). All cultures were maintained under cool white fluorescent lights of approximately 35 μmol · m^{-2} · s^{-1} photon flux density under a 16 h/8 h cycle at (24 ± 2) ℃. Leaf explants were collected at 0 h, 6 h, 12 h, 1 d, and 3 d, frozen immediately in liquid nitrogen, and stored at −80 ℃.

2.2. PPO activity assay

Frozen leaf explants of *Phalaenopsis* (200 mg) were mixed with 2 mL pre-cooled 0.2 mol · L^{-1} sodium phosphate buffer (pH 8.0) containing 0.1% TritonX-100. The homogenate was centrifuged at 12 000 × *g* for 10 min at 4 ℃. The supernatant, referred to as crude enzyme extract, was collected and stored at −20 ℃. For the PPO activity assay, 0.2 mL crude enzyme extract was mixed with 3 mL 2 mol · L^{-1} phosphate buffer (pH 8.0) and 0.2 mL 2% catechol. The reaction was carried out at 32 ℃. The changes in absorbance at 310 nm were recorded with a spectrophotometer (UV6000PC, Shanghai Metash Instruments Co.) every 20 s for 2 min. PPO activity is expressed as units · mg^{-1} protein. One unit of PPO activity is defined as a change in absorbance value (OD) of 0.001 min^{-1} · mg^{-1} of enzyme. The protein content was determined by the Bradford method using bovine serum albumin (BSA) as the standard.

2.3. Total phenolics assay

Total phenolics were determined according to the procedure of Luo et al. (1999). Segments of leaf explants were homogenized in a ten-fold quantity (w/w) of methanol (pH 3.0) using a mortar and pestle and extracted for 24 h at 4 ℃. The homogenate was centrifuged at 12 000 × *g* for 10 min and the resulting supernatant was used for the determination of phenolic compound contents. Absorbance was detected at 280 nm using a UV-visible spectrophotometer (Shimadzu UV2450). The total phenolic content in the sample was calculated based on a standard curve for catechol.

2.4. RNA blot analysis of PPO expression

To detect the expression of *PPO*, total RNA was isolated in Trizol Reagent (Invitrogen, CA, USA) following the manufacturer's protocol. Each 10 μg sample of total RNA was separated on a 1% agarose denaturing formaldehyde gel, transferred to a Hybond-N + nylon membrane, and fixed by UV crosslinking. Hybridisation and detection were conducted following the manufacturer's instructions (DIG Northern Starter Kit, Roche, Mannheim, Germany).

2.5. Immunoblot analysis of PPO protein levels

Frozen leaf explants (0.5 g) were ground to a fine powder in liquid nitrogen and homogenized in 1 mL pre-cooled lysis buffer (Keygen Biotech, Guangzhou, China) containing 1 μL protease inhibitor, 10 μL dithiothreitol (DTT) (1 mol · L^{-1}), and 10 μL phenylmethanesulfonyl fluoride (PMSF) (100 mmol · L^{-1}). The homogenate was centrifuged at 14 000 × *g* for 10 min at 4 ℃. The supernatant, referred to as the crude enzyme extract, was used for immunoblot analysis. Soluble proteins (50 μg total protein) were separated on SDS-PAGE (12% acrylamide) gels, transferred to nitrocellulose membranes (Millipore Corporation, Billerica, MA, USA), and probed with anti-PhPPO polyclonal antibodies (1 : 2 000 in 5% nonfat dry milk). Immunocomplexes were detected with HRP-labelled goat anti-rabbit IgG antibody. The protein content was determined by the Bradford method using bovine serum albumin (BSA) as the standard.

2.6. In situ hybridization of PPO RNA

Primers were designed for cloning the complete coding sequence of *PPO* into the pGEM-T Easy vector for probe synthesis with RNA polymerases T7 and SP6 using a DIG RNA labelling kit (Roche). The explants were fixed for 1 h under vacuum on ice and incubated in fixative overnight at 4 ℃. Instead of xylene, Histo-clear was used for clearing, and before the detection reaction, the slides were treated with RNase and washed with low-stringency buffer (2× SSC). The probes were detected after 3 h of development in the dark, which produced sufficient signals for detection.

2.7. In situ immunostaining of PPO protein

Leaf samples were fixed at 4 ℃ and dehydrated in 30% (w/v) sucrose. Leaf cross-sections 20 μm in thickness were cut with a microtome (Sorvall MT-6000 ultramicrotome). Immuno-enzyme detection of extensins in the sections was performed using the streptavidin method. The sections were blocked with 5% BSA in PBS and incubated overnight with anti-PhPPO polyclonal antibodies (1 : 200 10 mmol · L^{-1} PBS containing 5% BSA, pH 7.2) at 4 ℃.The slides were rinsed three times with PBS and incubated with HRP-labelled goat anti-rabbit IgG antibody (1 : 200) (Bio Rad) at 37 ℃ for 20 min. Following triple washing with PBS, the sections were treated with DAB at room temperature for 10 min and washed with distilled water. The localization of PPO was examined under a microscope (Olympus IX-70, Japan). Sections treated with PBS/BSA solution without primary antibody were used as negative controls.

3. Results

3.1. Effect of CHX on browning in Phalaenopsis explants

On the third day of culture, the *Phalaenopsis* explants had not turned brown, and a browning rate was 0%. However, by the sixth day, all the explants were completely brown, the browning rate of 100%. By contrast, after 0.1 mg · mL^{-1} CHX was added to the culture medium, only 40% of the explants had turned brown on day six of culture. Moreover, the extent of browning was less severe under this treatment (Fig. 1). Therefore, treatment with CHX inhibited the browning of *Phalaenopsis* explants.

3.2. Variation in PPO activity during the early stage of browning in Phalaenopsis explants

To determine the effect of PPO on the early browning stage of *Phalaenopsis* explants, we used samples after up to three days of culture for subsequent analysis. The PPO activity did not significantly change in explants cultured for 6 h and 24 h compared to the control. By day three, PPO activity had significantly increased. After treatment with CHX, followed by culture for 24 h, the PPO activity significantly decreased. Therefore, CHX treatment significantly inhibited PPO activity (Fig. 2).

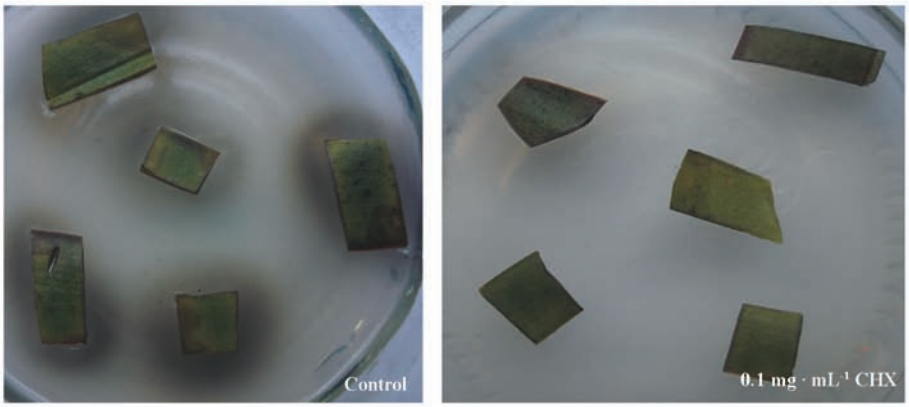

Fig. 1 Effects of CHX on *Phalaenopsis* leaf explant browning after 6 days culture

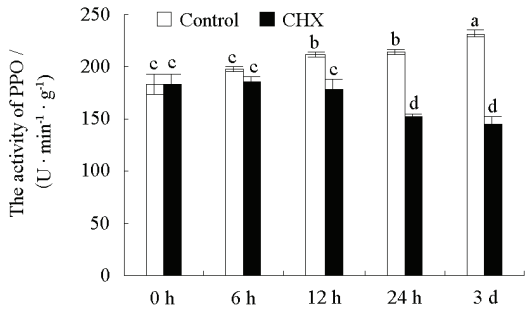

Fig. 2 Effect of CHX on PPO activity during *Phalaenopsis* leaf explant browning

As shown in Fig. 3, the total contents of phenolic compounds varied during the early stage of browning in *Phalaenopsis* explants. The total phenolic content significantly increased at 6 h compared to the control. However, by 12 h of culture, the total phenolic content decreased. The variations in total phenolic contents in CHX-treatment explants were consistent with those of the control. As the time in culture increased, the total phenolic content of CHX-treated explants continued to increase, exceeding that of the control.

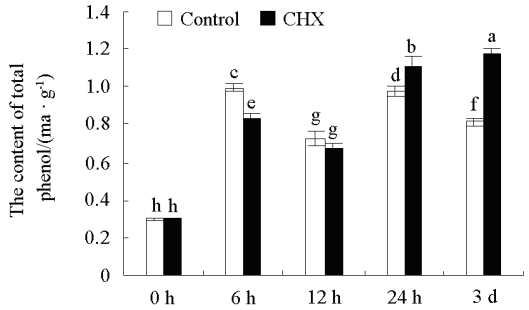

Fig. 3 Effect of CHX on total phenolic compound contents during *Phalaenopsis* leaf explant browning

3.3. Changes in PPO expression in the early stage of explant browning

PPO expression was not detected in explants cultured for 0 h. However, after 6 h of culture, *PPO* expression in the *Phalaenopsis* explants increased, with higher expression detected at 12 and 24 h of culture. However, by the third day of culture, *PPO* expression in the explants decreased to almost undetectable levels. After CHX treatment, we detected *PPO* expression in *Phalaenopsis* explants cultured for 6 h. However, the expression level was lower than that of the control; subsequently, *PPO* expression was nearly undetectable (Fig. 4). These results indicate that CHX treatment inhibited *PPO* expression and that *PPO* expression was initiated at 6 h of culture; this affected the subsequent browning in *Phalaenopsis* explants.

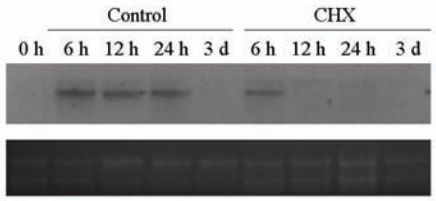

Fig. 4 Northern blot analysis of PPO protein in *Phalaenopsis* before explant browning

3.4. Changes in PPO protein levels in explants in the early stage of browning

PPO contents in *Phalaenopsis* explants cultured for 24 h and 3 d exceeded that of explants cultured for 0 h (Fig. 5). However, some PPO was detected in explants cultured for 0 h. These results indicate that in *Phalaenopsis* explants, some PPO is not produced by induction of *PPO* transcription, but it is already present in the explants. After treatment with the protein synthesis inhibitor CHX, PPO levels decreased after 6 h of culture, PPO levels increased slightly in explants cultured for 12 h but reached a minimum value in explants cultured for 3 d.

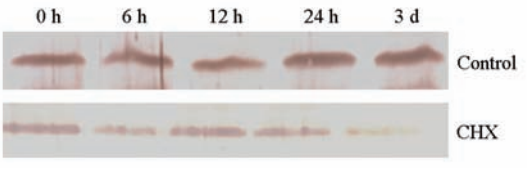

Fig. 5 Western blot analysis of PPO protein in *Phalaenopsis* before explant browning

3.5. Distribution of PPO protein in Phalaenopsis explants at the early stage of browning

We analysed the distribution of PPO protein at each stage and examined the effect of CHX treatment on the distribution of PPO in explant tissues (Fig. 6). The results indicate that PPO was mainly found in the vascular bundles of explant tissue. The distribution of PPO protein at 6 h, 24 h, and 3 d of culture is consistent with that observed at 0 h. The PPO protein content increased in explants cultured for 24 h and 3 d, and the explants exhibited intense staining. No significant differences in the distribution of PPO were detected between CHS-treated explants and the control.

3.6. Distribution of PPO mRNA in Phalaenopsis explants at the early stage of browning

Consistent with the RNA gel blot analysis described above, *in situ* hybridisation detected no *PPO* expression in explants cultured for 0 h (Fig. 7). However, *PPO* expression significantly

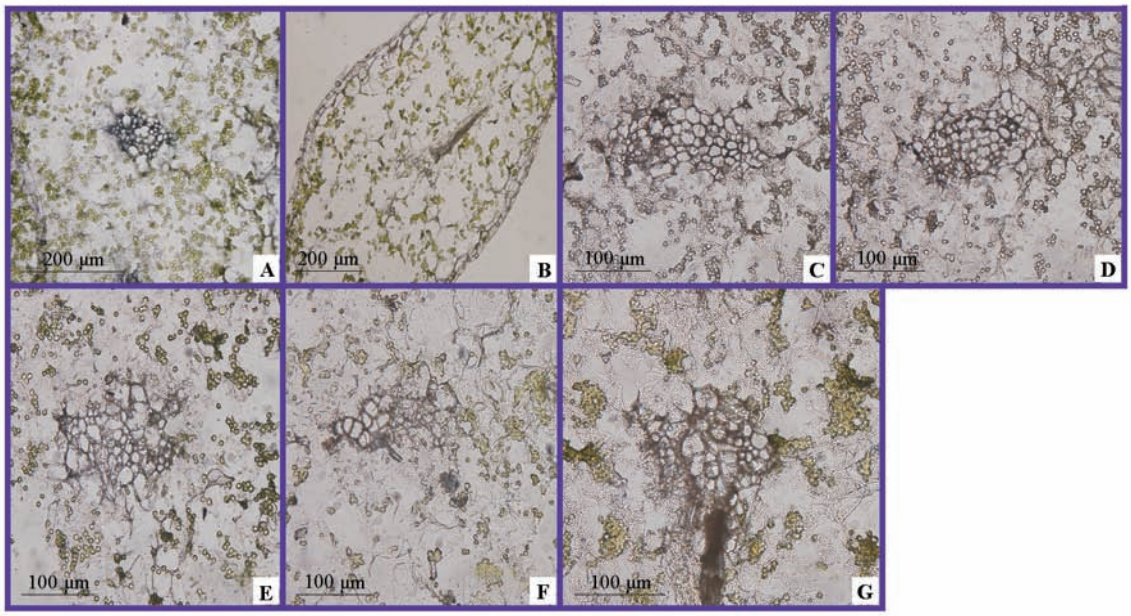

Fig. 6 Localization of PPO protein in *Phalaenopsis* leaf explants

A. Control; B. Explant cultured for 6 h; C. Explant cultured for 24 h; D. Explant cultured for 3 d; E. Explant treated with CHX and cultured for 6 h; F. Explant treated with CHX and cultured for 24 h; G. Explant treated with CHX and cultured for 3 d.

DAB stained PPO protein shows brown colour.

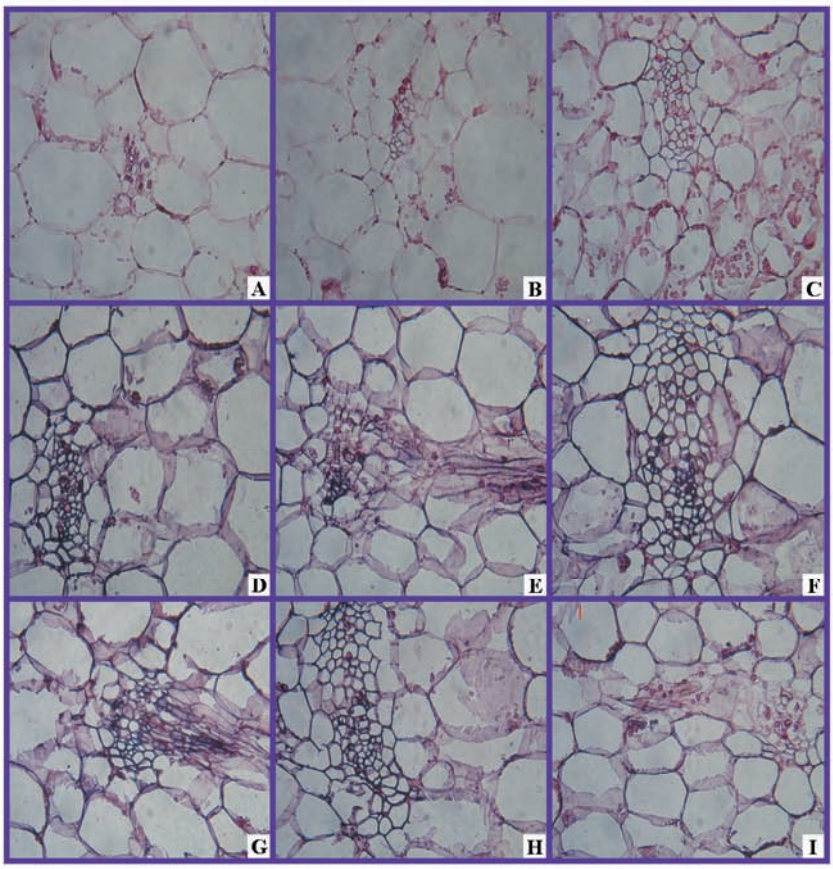

Fig. 7 *In situ* hybridisation analysis of the expression of the *PPO* gene in the early browning stage of *Phalaenopsis* explants

A. Negative control group; B. Blank control; C. Explants cultured for 0 h; D. Explants cultured for 6 h; E. Explants cultured for 24 h; F. Explants cultured for 3 d; G. CHX-treated explants cultured for 6 h; H. CHX-treated explants cultured for 24 h; I. CHX-treated explants cultured for 3 d.

increased in *Phalaenopsis* explants cultured for 6 h and the signal for the presence of the *PPO* mRNA was observed in the cytoplasm and vascular bundles after staining. The signal in the vascular bundles was darker than the signal in the cytoplasm. The explants cultured for 6 h showed stronger staining for *PPO* mRNA than did the explants cultured for 24 h and 3 d. Like untreated plants, in CHX-treated explants, *PPO* expression was detected in both the cytoplasm and vascular bundles. *PPO* expression in CHX-treated explants decreased on the third day of culture. *In situ* hybridisation revealed that *PPO* expression in the cytoplasm and vascular bundles of *Phalaenopsis* explants significantly increased at 6 h of culture.

4. Discussion

When plants are injured, reactive oxygen species bursts occur, which affect membrane integrity (Veltman et al., 2003; Franck et al., 2007; Cascia et al., 2013) and result in a loss of cellular compartmentalization. The accumulation and oxidation of phenolic compounds causes enzymatic browning (Di Guardo et al., 2013). Phenolics released by injury act as signalling molecules and induce increases in PPO levels through feedback regulation. Under these conditions, *PPO* expression and PPO protein levels increase (Thipyapong and Steffens, 1997; Constabel et al., 2000; Kruzmaneet al., 2002; Koussevitzky et al., 2004; Wang et al., 2008; Li et al., 2014); these processes are correlated with browning (Saltveit et al., 2005; Wahler et al., 2009). In the current study, we found that increased PPO activity in browning explants was accompanied by significant increases in total phenolic compound contentsin explants after 6 h of culture. Indeed, previous studies also demonstrated that increased phenol oxidase activity results in explant browning and that the degree of explant browning is positively correlated with increases in the levels of phenolic compounds. We previously determined that the vascular bundles of browning tissues are filled with tannin. Increasing antioxidant capacity to alleviate oxidative damage in freshly cut lotus root slices inhibits tissue browning (Sun et al., 2015). Moreover, reducing polyphenol biosynthesis and inhibiting the phenylpropanoid pathway repress browning (Hisaminato et al., 2001; Tanaka et al., 2011; Jones and Saxena, 2013). We previously demonstrated that genes involved in polyphenol biosynthesis and the phenylpropanoid pathway are upregulated in browning explants (Xu et al., 2015). Finally, Liu et al. (2013) found that genes involved in phenolic biosynthesis are upregulated in pear fruit during browning.

Studies have shown that the expression of *PPO* could be induced by wound injury (Constabel et al., 2000; Haruta et al., 2001; Shetty et al., 2011; Jiang et al., 2012; Boeckx et al., 2015). The current results indicate that *PPO* expression significantly increased in *Phalaenopsis* explants at 6 h of culture. This finding suggests that during *Phalaenopsis* explant culture, mechanical injuries caused by processes such as excision of the tissue from the parent plant induce the expression of *PPO*. With prolonged culture, *PPO* mRNA and PPO protein levels remained strong, indicating that they had the effect on the subsequent occurrence of severe browning. *Phalaenopsis* explants cultured for 0 h exhibited very low PPO activity; however, immunoblot analysis revealed that *Phalaenopsis* explants cultured for 0 h had high PPO protein contents, consistent with the previous studies that PPO might be present in an inactive pro-enzyme form and had been synthetic at the early stage of leaves development (Haruta et al., 2001; Sullivan et al., 2004). We previously found that sodium dodecyl sulphate (SDS) treatment increases PPO activity (Chen et al., 2009). Other studies have also indicated that the latent form of PPO is available and inducible by injury, protease treatment, SDS, and other treatments. Moreover, *PPO* exhibits constitutive, inducible expression and *de novo* biosynthesis (Thipyapong and Steffens, 1997; Mazzafera and Robinson, 2000; Haruta et al., 2001; Wang and Constabel, 2003; Lee et al., 2009). These results suggest that browning of *Phalaenopsis* explant during tissue culture results from release of pre-existing PPO enzyme and synthesis later.

CHX is a protein synthesis inhibitor in eukaryotic organisms. In the current study, the addition of 0.1 mg · mL^{-1} CHX to the culture medium inhibited the browning of *Phalaenopsis* explants, indicating that newly synthesised PPO protein participates in explant browning. We analysed the effects of CHX treatment on *Phalaenopsis* explants during the browning process, finding that this treatment inhibited *PPO* gene expression and reduced PPO protein levels compared to the control.

Analysis of the distribution of *PPO* mRNA and PPO protein in the early stage of explant browning in *Phalaenopsis* suggested that these molecules were concentrated in vascular bundles. CHX treatment did not affect the distribution of the *PPO* mRNA or PPO protein, although it decreased their levels. These results indicate that the browning of *Phalaenopsis* explants occurs during the early stage and mainly affects the vascular bundles. Using a chemical localization method, Wang et al. (2007) found that PPO is concentrated in the vascular bundle ring. They also determined that vascular bundles in *Phalaenopsis* explants that have undergone browning are destroyed and filled with tannin and that phenolic compounds are mainly deposited in vascular bundles during explant browning (Xu et al., 2006, 2015). The high levels of *PPO* mRNA and PPO protein in the vascular bundles of *Phalaenopsis* explants indicate that browning is closely related to vascular bundle injury. Vascular bundle injury hinders the transpiration of water and nutrients and is the underlying cause of explant death.

5. Conclusions

This study represents the first analysis of the effects of PPO on the early stage of browning in *Phalaenopsis* explants from a molecular perspective. The results show that *in vivo*, PPO accumulation initiates at the early stage (6 h) of explant browning in *Phalaenopsis* and newly synthesised PPO protein is involved in the occurrence of subsequent browning. PPO protein and *PPO* mRNA were mainly distributed in the vascular bundles of the explants. CHX treatment merely reduces the frequency of the occurrence of explant browning and inhibits the expression of *PPO* gene and PPO protein in *Phalaenopsis* explants, but has no effect on the distribution of PPO in tissues.

Acknowledgements

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News

Welcome to the Second Asian Horticultural Congress 2016

The Second Asian Horticultural Congress will be opened in Chengdu, Sichuan of China during September 26 to 28, 2016. This time, it will be co-hosted by International Society for Horticultural Science, Chinese Society for Horticultural Science, Korean Society for Horticultural Science and Japanese Society for Horticultural Science, and co-organized by Sichuan Academy of Agricultural Science and Chengdu Academy of Agricultural Science. With the first Congress held in 2008 in Cheju, Korea, the Asian Horticultural Congress was initiated by societies for horticultural science from China, Japan and Korea.

Asia is endowed with plenty of important horticultural resources, and is the area of origin for many horticultural plants. Located across tropical, subtropical, temperate, and frigid zones, Asia has complicated climate, diversified ecosystems as well as profound farming culture. Therefore, on this land, many horticultural modes, models and technologies of production with considerate regional characteristics and cultural features are formed. And since the beginning of the 21th century, new developments of Asian horticultural science are continuously made both in basic research such as various omics, physiology and genetics and in the study of applied technologies promoting high yield, highly-efficient and sustainability of production, along with constant innovation in science and technology around the world, especially in biotechnology. However, no doubt that the horticultural industry of Asian countries is facing challenges in respects of resource shortage, rising of labor cost, pollution of environment, etc. To meet these challenges, we need to exchange and collaborate more than ever. As an exchange platform this Congress will provide fine opportunities of sharing knowledge, thoughts and experience, and the opportunities for cooperation to colleagues and friends engaging in scientific research on and production of fruit trees, vegetables, and flowers.

Finally, the Organizing Committee of the Second Asian Horticultural Congress extends warm welcome to all researchers, teachers, students, executives and relevant professionals working in horticultural research and industry. Your presence are cordially requested. http://ciccst.org.cn/ahc2016/

Sincerely yours,

Yongchen Du
President
Chinese Society for Horticultural Science